Video Article

Generation of Recombinant Influenza Virus from Plasmid DNA

Luis Martínez-Sobrido[1], Adolfo García-Sastre[2]

[1]Department of Microbiology and Immunology, University of Rochester
[2]Departments of Microbiology and Medicine, and Global Health and Emerging Pathogens Institute, Mount Sinai School of Medicine

Correspondence to: Luis Martínez-Sobrido at Luis_martinez@urmc.rochester.edu

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Abstract

Efforts by a number of influenza research groups have been pivotal in the development and improvement of influenza A virus reverse genetics. Originally established in 1999 [1,2] plasmid-based reverse genetic techniques to generate recombinant viruses have revolutionized the influenza research field because specific questions have been answered by genetically engineered, infectious, recombinant influenza viruses. Such studies include virus replication, function of viral proteins, the contribution of specific mutations in viral proteins in viral replication and/or pathogenesis and, also, viral vectors using recombinant influenza viruses expressing foreign proteins [3].

Protocol

1. Influenza virus rescue transfection

Influenza A virus belongs to the *Orthomyxoviridae* family of negative-stranded RNA enveloped viruses. The influenza A virus genome consists of eight different RNA genes of negative polarity that encode, at least, 11 viral proteins (Figure 1) [4]. We will focus, in this report, on the rescue of one of the most common laboratory strain, influenza A/PR/8/34, [5] using ambisense plasmids (pDZ) containing the 8 influenza A/PR/8/34 viral segments (Figure 2).

For the rescue of recombinant influenza viruses from plasmid DNA , we recommend 3 independent transfections per each recombinant virus. If more than one recombinant virus rescue is attempted, scale the following steps accordantly to the number of viruses to be rescued. The following transfection and infection protocol is established for 6-well-plates. A schematic representation of the protocol is illustrated in Figure 3.

1. OptiMEM-Lipofectamine 2000 (LPF2000) mixture: Prepare 250 µl of OptiMEM media and 6-8 µl of LPF2000 per transfection. Incubate for 5-10 minutes at room temperature (RT). Meanwhile, prepare the plasmid transfection mixture.
2. Plasmid transfection mixture: Prepare the plasmid transfection cocktail in 50 µl of OptiMEM media. We usually use 1 µg of each influenza DNA plasmid per rescue. Add 1 µl of the pDZ plasmids (at 1 µg/µl) PB2, PB1, PA, HA, NP, NA, M, and NS to a tube containing 50 µl of OptiMEM media.
3. OptiMEM-LPF2000-DNA plasmid mixture: Add 250 µl from step 1.1 into the influenza DNA plasmid transfection mixture (step 1.2). Incubate this mixture for 20-30 minutes at RT. Meanwhile, prepare suspensions of 293T and MDCK cells for transfection.
4. Preparation of 293T/MDCK co-culture: Before starting, bring the PBS 1X, DMEM 10%FBS 1% PS media, and EDTA-trypsin mixture to 37°C. The density of the cells should be at 80-90% confluence the day of transfection. Usually, one confluent 100 mm dish of 293T and one confluent 100 mm dish of MDCK cells can be used for 10-12 rescues. We are going to use 250 µl of cells per well. Both cell lines will be resuspended in a total of 3 ml of DMEM 10%FBS 1%PS.
    - Carefully resuspend each cell line in 10 ml of DMEM 10%FBS 1%PS in a 15 ml centrifuge tube. You will have one tube for 293T cells and one tube for MDCK cells.
    - Resuspend the 293T cells in 3 ml of DMEM 10%FBS 1%PS and when resuspended, deliver the 3 ml to the MDCK cells to resuspend those cells. This will give you the mixture of 293T and MDCK cells to be used for your co-culture.
    - Add 250 µl of the 293T/MDCK cells per well (10-12 6-well wells).
5. After 20-30 minutes RT incubation (step 1.3), add 1 ml of DMEM 10%FBS 1%PS to the OptiMEM-LPF2000-influenza DNA plasmid mixture.
6. Add the 1.3 ml (step 1.5) into the wells with the 250 µl of 293T/MDCK cells (step 1.4).
7. Gently shake the 6-well-plate and let the transfection incubate overnight (ON) in the incubator at 37°C and 5% CO_2.
8. Next day, approximately 16-24 hours post-transfection, change the transfection media and incubate the transfected cells in DMEM 0.3%BA 1%PS containing 1 µg/ml of TPCK-trypsin for 48 hours.
9. After 48 hours of changing the media, transfer the supernatant from the transfected cells into a microcentrifuge tube.
10. Centrifuge the tissue culture supernatant in a microcentrifuge for 1-2 minutes, 13,000 rpm.
11. Infect fresh MDCK cells in 6-well plates (plated the day before) or 10-day-old chicken embryonated eggs with 200 µl of centrifuged tissue culture supernatants from step 1.10. Incubate the cells and/or eggs at 37°C for 2-3 days.
    1. Infection of 10-day-old chicken embryonated eggs: All procedures to infect chicken embryonated eggs are performed under sterile conditions.
        1. Candle the 10-day-old eggs using a light-candling box to see the interface between the air sac and the allantoic cavity. Make a pencil mark on the interface border.
        2. With a 5 ml syringe needle make a hole in the eggshell.
        3. With a 1 ml syringe, infect each egg with 200 µl of the tissue culture supernatants from step 1.10.
        4. Cover the hole in the eggshell with melted wax using a cotton swab.
        5. Incubate the infected eggs at 37oC for 2-3 days.

2. Infection of fresh MDCK cells: The day before passage the tissue culture supernatant from the 293T/MDCK co-cultures, prepare 6-well plate dishes with MDCK cells to reach 80-90% confluence next day. Usually, a confluent 100 mm tissue culture plate can be split into 6-8 wells. Wash the cells, twice, with PBS 1X, trypsinize and prepare the 6-well plates. Gently shake by hand the plates in order to have a uniformed distribution of the cells. Culture the cells, ON, in the 37°C incubator, 5% CO_2. Before infection, check the cells under the microscope to confirm a monolayer; then, proceed with the infection:
    - Wash cells, twice, with 1 ml of PBS 1X.
    - Infect with the 200 µl of centrifuged tissue culture supernatants for 1 hour at RT. Do not let the cells dry. Rock the 6-well-plate every 10 minutes.
    - After 1 hour of viral absorption, remove the infection media from the MDCK cells and add 2 ml of DMEM 0.3%BA 1%PS containing 1 µg/ml of TPCK-trypsin.
    - At 48-72 hours after passage, depending on the transfection efficiency and the virus load, a cytopathic effect (CPE) will be observed in the MDCK infected cells. CPE suggests a successful rescue. However, an HA assay (section 2) should still be performed to confirm the presence of the virus in the tissue culture supernatants.

12. Harvest allantoic fluid from infected chicken embryonated eggs: All procedures to harvest the allantoic fluid from infected eggs are performed under sterile conditions. Approximately 8-12 ml of allantoic fluid can be harvested from each 10-day-old-infected egg. Prior to harvesting the allantoic fluid, incubate the chicken eggs for 2 hours (or ON) at 4°C to kill the chicken embryo and coagulate the blood.
    - Wash the eggshells with 70% ethanol to establish sterile conditions.
    - Open the egg, carefully, over the air cavity by tapping with a spoon. Remove the broken eggshell with the help of forceps.
    - With a 1 ml needle, remove the allantoic membrane without breaking the egg's yolk.
    - Stabilize the chicken embryo with a spatula as you guide a 10 ml pipette into the allantoic fluid. Collect as much allantoic fluid as possible into a 15 ml centrifuge tube on ice in an ice bucket without breaking or collecting any of the egg's yolk. Use a 15 ml centrifuge tube for each egg.
    - Centrifuge for 5 minutes at 4°C and transfer the allantoic fluid (without taking pelleted red blood cells) to a fresh 15 ml centrifuge tubes.
    - Store the tubes containing the centrifuged allantoic fluid at 4°C until they are checked for the presence of rescued virus with a hemagglutination (HA) assay.

2. HA assay to confirm the rescue of recombinant influenza viruses

Hemagglutination assay (HA) is routinely used to detect the presence of rescued virus in MDCK tissue culture supernatants and/or the allantoic fluid of harvested eggs. Alternatively, immunofluorescence assays (IFA) can be also performed. Once an assay identifies the presence of rescued virus, the virus should be plaque purified and the genetic composition of the virus will be confirmed by RT-PCR and sequencing.

The presence of virus in the MDCK tissue culture supernatants and/or in the allantoic fluid from infected eggs can be determined macroscopically using HA of chicken (or another source) red blood cells (RBC). The presence of virus induces hemagglutination of RBC while the absence of virus allows the formation of a red pellet in the bottom of the well (Figure 4). In the case of influenza virus, it is believed that approximately 10^3-10^4 plaque forming units (PFU) are required to give a positive signal in the HA assay; therefore, an IFA can be performed in parallel with the HA assay to confirm a true negative result. IFA with primary anti-influenza antibodies is more sensitive than the HA assay because less than 103-104 viruses can be detected with this technique. It is possible than supernatants or allantoic fluids that are HA-negative are positive by IFA. In this case, the virus should be amplified by passaging, again, in MDCK cells or in eggs. Allantoic fluid and/or tissue culture supernatants from the second passage should now be clearly positive in the HA assay.

HA assays are carried out in V-bottom 96-well plates. Negative (for instance, PBS 1X) and positive (tissue culture supernatants and/or allantoic fluid from an influenza virus infection) control samples should always be included in any HA assay to validate it.

- Dispense 50 µl of PBS 1X into each well of the V-bottom 96-well plate.
- Add 50 µl of the MDCK tissue culture supernatants and/or allantoic fluid from the infected eggs to the first well and, make 2-fold serial dilutions for the following wells. Discard the extra 50 µl from the last well.
- Add 50 µl of 0.5%-1.0% chicken red blood cells (prepared in PBS 1X) to each well.
- Incubate the V-bottom 96-well plate for 30-45 minutes (until a red dot is visible in the bottom of a negative control PBS sample) on ice. Read and interpretate the results as indicated in Figure 4.

3. Passage of tissue culture supernatants

A negative result in the HA assay may be a result of low transfection efficiency with low levels of virus being present in the tissue culture supernatants and/or allantoic fluid. Passage of these samples in fresh MDCK and/or embryonated eggs will allow amplification of the virus (as indicated in Figure 3) Infections are performed as previously described in section 1.11.2.

4. Representative Results

Successful influenza virus rescue will be confirmed by the presence of a positive HA assay (Figure 4). Additionally, the existence of CPE in cells infected with the tissue culture supernatants or with the allantoic fluid from eggs will suggest a positive viral rescue.

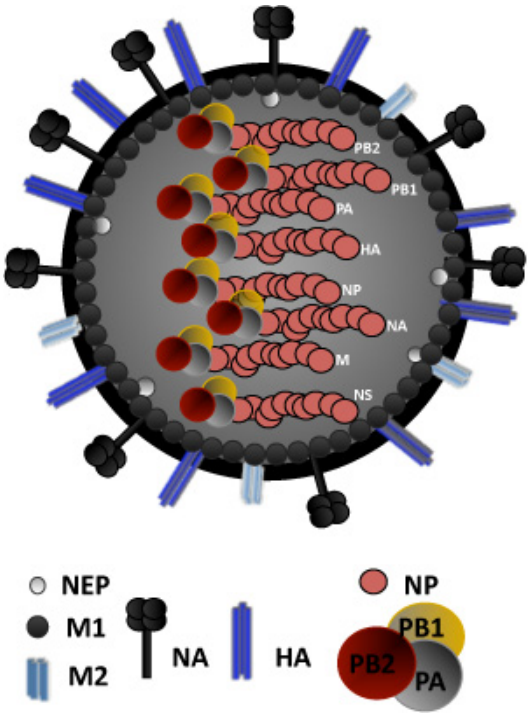

Figure 1. Influenza Virus structure: Influenza virus is surrounded by a lipid bilayer containing the two viral glycoproteins (HA, NA) and, also, the ion channel protein, M2. HA is the viral attachment protein, responsible for binding to sialic acid-containing receptors. NA is responsible for viral release from host cells. Underneath the lipid bilayer, is a protein layer composed of the inner surface envelope matrix protein 1, M1, which plays a role in virion assembly and budding and the nuclear exporting protein (NEP), required for nuclear export of viral ribonucleocapsids. The core of the virus is made of a ribonucleoprotein (RNP) complex, composed of 8 single-stranded negative RNA viral genes encapsidated by the viral nucleoprotein, NP. Associated with the RNP complex are the viral RNA-dependent RNA polymerase subunits PA, PB1, and PB2. The non-structural proteins NS1 and PB1-F2, encoded by the RNA segments NS and PB1, respectively, are not part of the virion structure.

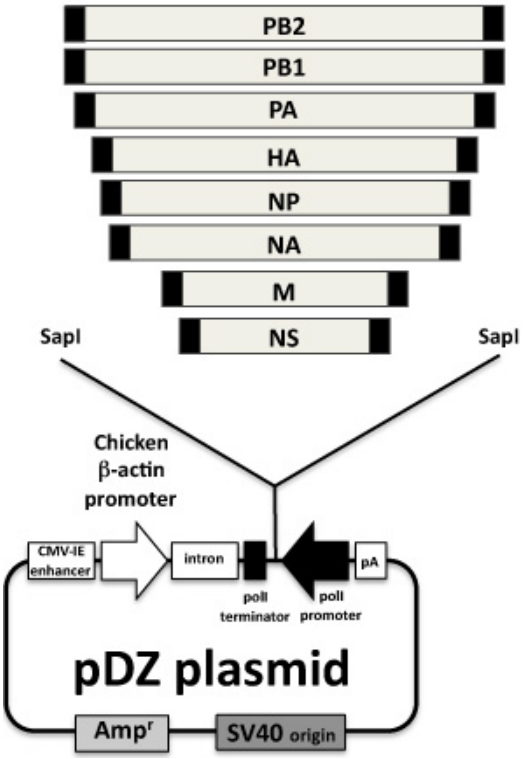

Figure 2. Influenza virus rescue plasmids: The eight influenza virus genes cloned into the ambisense plasmid pDZ are indicated. pDZ plasmid [6], derived from the protein expression plasmid pCAGGs [7], is a bidirectional plasmid vector with a human RNA polymerase I promoter and a mouse terminator sequence that encodes the negative sense genomic RNA; in opposite orientation to the polymerase I unite, a polymerase II transcription cassette (chicken β-actin promoter and polyA) encodes the viral proteins from the same viral gene. cDNAs from each viral segment are generated by RT-PCR with forward and reverse primers containing the SapI restriction endonuclease site and the noncoding regions of each segment (black boxes at the end of the viral genes). The PCR product is cloned into the pDZ digested with Sap-I.

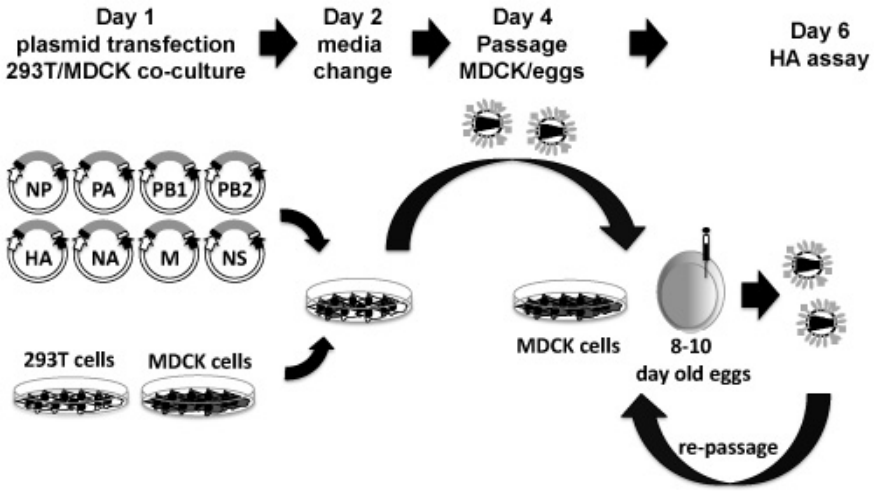

Figure 3. Eight-plasmid-based influenza rescue system: pDZ plasmids containing the 8 influenza viral genes are co-transfected, in suspension, in 293T-MDCK cells co-cultures (day 1). Twenty-four hours post-transfection, media without FBS but containing TPCK/trypsin is replaced (day 2). Forty-eight hours after changing media, tissue culture supernatant is harvested and used to infect MDCK or 10-day-old embryonated chicken eggs (day 4). 48-72 hours post-amplification, tissue culture supernatants from MDCK infected cells or allantoic fluid from eggs are harvested and assayed for presence of virus by HA (day 6). If no virus is detected, the same supernatants and/or allantoic fluids can be re-passaged into fresh MDCK cells and/or embryonated eggs.

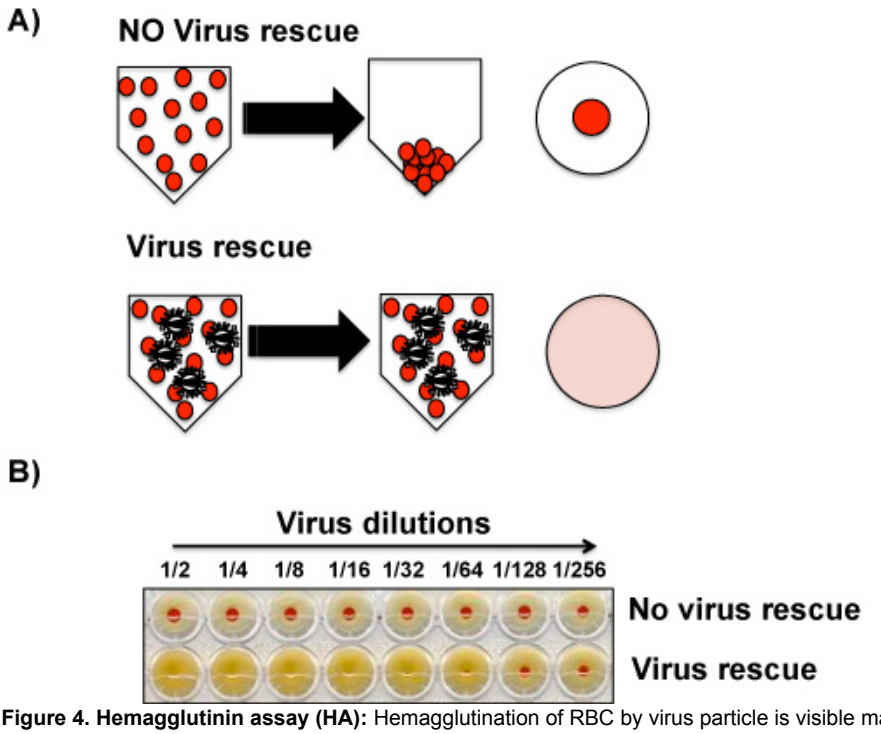

Figure 4. Hemagglutinin assay (HA): Hemagglutination of RBC by virus particle is visible macroscopically and is the basis to detect viral particles in tissue culture supernatants or/and allantoic fluids. Although the HA assay does not discriminate between viral particles that are infectious and particles that are degraded and no longer able to infect cells, the assay is a good indicator of presence of virus in samples. A) Absence (top) of presence (bottom) of virus in the biological samples is determined by presence of RBC in the bottom of the plate or their absence, respectively. B) A representative result from an HA assay with no detectable levels of virus (top) or presence (bottom) of virus is shown.

Discussion

Rescue of recombinant influenza viruses from plasmid DNA is a simple and straightforward process once the protocol is routinely performed in the laboratory, but in the beginning, multiple things can go wrong. It is imperative to have good plasmid preparation to generate the virus. Proper maintenance of the cell lines (293T and MDCK) is crucial for a successful viral rescue. Traditionally, a genetic tag is inserted into an influenza gene-encoding plasmid, by silent mutagenesis. Introduction of this silent mutation(s) and creation of, for instance, a novel restriction enzyme site is used to distinguish between wild-type and recombinant influenza virus by enzyme digestion. Therefore, after amplification of the plaque purified recombinant virus, RT-PCR and sequencing approaches should be performed to verify the nature of the rescued virus. The development of these reverse genetics techniques and successful rescue of recombinant influenza viruses from plasmids will allow you to answer specific questions about the biology of the virus.

Disclosures

Mount Sinai School of Medicine has intellectual property rights in the area of recombinant influenza viruses, and AG-S is an inventor in this intellectual property.

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